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THE BACTERIAL FLORA OF MILK HELD AT LOW TEMPERATURES.*

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The value of milk and cream for human food, either alone or as constituents of more elaborate dishes, has led to efforts for their preservation for considerable periods of time. Such preservation is necessary in order to furnish a supply for places where the fresh product cannot be obtained, as on ocean steamships, or for a time of abnormal demand, such as that occasioned by the Fourth of July. The bacterial changes undergone by milk during its preservation at low temperatures have been made the subject of considerable study by Conn and Esten,[1] and by Pennington,[2] these investigators being preceded by Havemann,[3] Schmidt-Nielson,[4] Schmelk,[5] Conradi and Vogt,[6] Fischer,[7] and Muller,[8] who showed that certain bacteria can grow at a temperature as low as 0° C. The investigations of these earlier scientists consisted mainly in keeping various inoculated menstra at about 0° C. and making quantitative bacterial determinations at stated times. Their conclusions were that, altho growth does take place, it is comparatively slow.

Conn and Esten showed that at 1° C. there is a period of from six to eight days during which there is no increase in the total number of bacteria in milk, and that when growth does start at this temperature the lactic bacteria fail to predominate over the other species, so that all types develop. They concluded that milk is not necessarily wholesome because it is sweet and that the species developing at this low temperature are more apt to be injurious than species developing at 20° C. Pennington concluded that at the low temperature (29–32° F.) employed in her experiments there were constantly present bacteria which formed acid and bacteria which acted on protein, the former

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[1] *Report Storrs Agri. Exper. Sta.*, 1904, p. 27.

[2] *Jour. Biol. Chem.*, 1908, 4, p. 353.

[3] *Ueber das Wachstum von Mikroorganismen bei Eiskrank Temperature*, Inaug. Dis., Rostok, 1894.

[4] *Centralbl. f. Bact.*, 1902, 9, Abt. II, p. 145.

[5] *Ibid.*, 4, Abt. I, p. 545.

[6] *Ztschr. f. Hyg.*, 1899, 331, p. 287.

[7] *Deut. med. Wchnschr.*, 1893, 19, p. 598.

[8] *Arch. f. Hyg.*, 1903, 47, p. 127.

being in relatively smaller numbers and the latter in relatively higher numbers than in milk kept at a higher temperature. Chemical study showed that the casein was rapidly digested until more than 50 per cent of it was changed to soluble compounds apparently caseoses, amino acids, and probably peptones.

The experiments herein detailed were undertaken with the intention of studying the activity of the bacterial flora of milk and water kept at both −9° and 0° C. Two milks were used, one, referred to as Barn milk, was supplied by the university herd; while the other, designated Dairy milk, was obtained at the university creamery, being a mixt milk supplied by the farmers about the city. The Barn milk was considered to be the best milk obtainable; it is produced under very good conditions and the average bacterial count is about 3,000 per c.c., with a low percentage of acid formers. The Dairy milk was taken because it was considered to be a fair dairy sample produced under average conditions; the bacterial count is quite variable with a rather large percentage of acid formers. The water used was that supplied to the various laboratories at the University of Wisconsin. The source is a lake of about 30 square miles in area.

At the beginning of the experiment the only medium used was a 2 per cent lactose agar having an acidity of 0.5 per cent, the plates being incubated at 37° C. Later the idea of using a medium somewhat more differentiating in character presented itself and a 2 per cent lactose gelatin with an acidity of 1 per cent was employed, the gelatin plates being incubated at 12°–15° C. These two media were then employed to the close of the experiment.

The containers used were sterile 8 oz. bottles. These were filled to within 25 c.c. of their capacity, in the case of the Barn milk immediately after the milk was drawn, while in the case of the Dairy milk at once upon arrival of the milk at the creamery. The bottles were then stoppered with sterile corks and sealed with paraffin. The specimens so prepared were at once packed in ice, repacked in ice and shavings in about two hours, and shipped to Waterloo 22 miles distant, where they were immediately freighted to the cold storage plant, unpacked, and stored in their respective cold rooms.

In order to see what effect the transportation would have on the bacterial content of the specimens, one bottle of each kind of milk was plated before repacking in ice and shavings, and one bottle of each was brought back from Waterloo the same day and plated. These results are given in the following table:

TABLE 1.
EFFECT OF SHIPMENT ON BACTERIAL CONTENT OF MILK.

	No. per c.c. before Repacking	No. per c.c. after Transportation
Barn milk..................	3,500	5,300
Dairy milk.................	130,000	175,000
Water.....................	68	29

This indicates that the transportation has only an insignificant effect on the bacterial content of the milks in question.

SPECIMENS KEPT AT -9° C.

The specimens were placed in the cold rooms August 6, 1908, and from that time on bottles were brought to the laboratory and plated at various times with the following results:

TABLE 2.
BACTERIAL CONTENT AT VARIOUS INTERVALS OF SPECIMENS HELD AT -9° C. PLATED ON LACTOSE AGAR AND INCUBATED AT 37° C.

Date	Age of Milk in Days	Water	Dairy Milk	Barn Milk
8-6-'08...........	0	68	130,000	3,500
8-12-'08..........	6	15	108,500	6,400
8-21-'08..........	15	16	95,500	5,850
8-26-'08..........	20	9	92,000	5,250
9-11-'08..........	36	9	111,500	3,050
9-11-'08..........	36	17	100,500	1,200
10-19-'08..........	74	26	127,000	5,000
11-20-'08..........	106	16	63,750	1,950
1-13-'09..........	160	5	51,000	....

TABLE 3.
BACTERIAL CONTENT AT VARIOUS INTERVALS OF SPECIMENS HELD AT -9° C. PLATED ON PLAIN GELATIN AND INCUBATED AT 12°–15° C.

Date	Age of Milk in Days	Water	Dairy Milk	Barn Milk
8-21-'08..........	15	14	8,600,000	3,400
8-26-'08..........	20	28	3,200,000	6,250
9-11-'08..........	36	30	600,000	2,650
9-11-'08..........	36	26	650,000	3,600
10-19-'08..........	74	16	145,000	5,750
11-20-'08..........	106	10	65,000	3,200
1-13-'09..........	160	5	57,000	....
2-25-'09..........	203	16	25,300	1,650

Milks.—All the bottles kept at -9° C. remained in a frozen condition until brought to the laboratory and here they were thawed just previous to plating. After six days the casein and fat were thrown into clumps that could not be again incorporated into the milk serum; this condition caused considerable difficulty in sampling and introduced an exceptionally large experimental error. At this time the odor and taste in both specimens were those of good milk and these conditions persisted for 36 days, when there was a decided watery taste in each case, while on standing over night, after being melted, the casein would be at the bottom of the bottle with a watery layer above. After 74 days the Dairy milk was slightly off in flavor altho the Barn milk was not, but after 106 days, both specimens were off in odor and taste; the odor was not suggestive of putrefaction, but was decidedly undesirable. The number of organisms developing at 37° C. on agar remains fairly constant, the variations evidenced being insufficient to allow of any definite conclusions. After 36 days storage, duplicate bottles were brought to the laboratory and plated and the results given in the table show that the differences between bottles of the same milk are negligible and quite within the limit of experimental error.

The lack of gelatin counts early in the experiment leaves a considerable gap in the results, but the data show that while the number of organisms in Barn milk developing on this medium remains fairly constant, the number in Dairy milk diminishes considerably.

The acidity of each specimen of milk was determined as soon after reaching the laboratory as possible and the data are presented in the following table:

TABLE 4.

ACIDITY OF MILKS HELD AT −9° C.

Date	Age of Milk in Days	Dairy	Barn
8- 6-'08	0	.16	.18
8-12-'08	6	.15	.18
8-21-'08	15	.16	.18
8-26-'08	20	.15	.18
9-11-'08	36	.14	.17
9-11-'08	36	.14	.16
10-20-'08	74	.13	.15
11-20-'08	106	.11	.09
1-13-'09	160	.11	.10
2-25-'09	203	.10	.10

From these results we see that there is a decrease in acidity and this was, of course, due either to a purely chemical, or to a biochemical change. The latter possibility presupposes a change in the bacterial flora of the milk; such a change cannot be quantitative, as has been shown by our determinations, altho there is, of course, the possibility that the determining organisms in this reaction-change do not develop on the media used. It seems more probable that the change is purely chemical and altho as yet we have no data to prove this hypothesis, experiments are at present under way which will furnish evidence along this line.

The milks examined after 36 and 203 days storage were left at room temperature for two and four days respectively and then titrated again, with the idea of finding out whether or not there would be an increase in acidity.

TABLE 5.

INCREASE IN ACIDITY DUE TO INFLUENCE OF HIGHER TEMPERATURE.

Sample	9–11–'08	9–13–'08	2–25–'09	3–3–'09
Barn....................	.17	.43	.10	1.71
Dairy....................	.14	.62	.10	1.11

This table shows that when the milks are exposed to room temperature the acid-forming bacteria again gain the supremacy over the other forms and the acidity of the specimen increases.

On the milks brought to the laboratory after 203 days storage, total nitrogen and water soluble nitrogen determinations were made. Because of the physical condition of the milks it was a difficult matter to obtain a sample and the method finally decided upon was to pipette off 5 c.c., using a pipette with a 3 mm. opening, for the total nitrogen determination. In the case of the water soluble nitrogen a 10 c.c. sample was placed in a beaker, diluted to 50 c.c. with water, a small amount of acetic acid added, and the resulting material heated on a water bath for some time, after which the soluble nitrogen compounds were allowed to filter into a Kjeldahl flask. The results given in the following table are the averages of duplicates which agreed very well:

TABLE 6.

NITROGEN DISTRIBUTION IN MILKS HELD AT −9° C.

	Percentage Total Nitrogen	Percentage Insoluble Nitrogen	Percentage Soluble Nitrogen	Percentage of Total Nitrogen Present as Soluble Nitrogen
Barn −9°..........	.5375	.4409	.0966	17.97
Dairy −9°.........	.5161	.4006	.1155	22.38

In comparison with fresh milk, which shows about 10 per cent of its nitrogen in a soluble form, we see here a percentage almost double that in fresh milk. This is probably due to enzyme action as this interpetation agrees very well with the data obtained at the Wisconsin Experiment Station during the study of galactase.[1]

Water.—The data obtained in the case of the water held at −9° C. shows that at this temperature there is no increase in the number of organisms developing on either lactose agar or gelatin.

SPECIMENS KEPT AT 0° C.

These bottles were also refrigerated August 6, 1908, and were plated on the same days as the specimens kept at −9°. The results are given in the following tables:

TABLE 7.

BACTERIAL CONTENT AT VARIOUS INTERVALS OF SPECIMENS HELD AT 0° C. PLATED ON LACTOSE AGAR AND INCUBATED AT 37°.

	Age of Milk in Days	Water	Dairy Milk	Barn Milk
8–6–'08	0	68	130,000	3,500
8–12–'08	6	12	72,500	4,050
8–21–'08	15	22	633,500	52,900
8–26–'08	20	21	3,230,000	1,240,000
9–11–'08	36	14,160	34,950,000	4,800,000
10–19–'08	74	33,300	91,500,000	36,500,000
11–20–'08	106	8,000	39,750,000	192,500,000
1–13–'09	160	2,300	32,650,000	361,000,000

TABLE 8.

BACTERIAL CONTENT AT VARIOUS INTERVALS OF SPECIMENS HELD AT 0° C. PLATED ON PLAIN GELATIN AND INCUBATED AT 12°–15° C.

	Age of Milk in Days	Water	Dairy Milk	Barn Milk
8–21–'08	15		10,950,000	252,000
8–26–'08	20		53,467,000	2,765,000
9–11–'08	36		160,000,000	39,000,000
10–19–'08	74	225,000	135,000,000	111,500,000
11–20–'08	106	165,500	88,000,000	229,500,000
1–13–'09	160		94,500,000	191,500,000
2–25–'09	203		65,500,000	183,000,000

Milks.—These samples never showed any ice formation, altho a dish of water standing beside the bottles developed a scum of ice. The specimens brought back after 36 days were the first to show an off flavor; this was but slight in the Barn milk, but in the Dairy milk it was very decided, the odor in each case, however, being about normal. On this date the bottle of Dairy milk showed a clumping of the casein, altho this was not as evident as in the milks at the lower temperature.

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[1] *Wisconsin Experiment Station, Twentieth Rept.*, p. 201.

The bottles brought back after 106 days both gave an appearance of digestion, and after shaking and drawing the cork there was a vigorous evolution of bad smelling gases.

The Dairy milk showed a steady increase in organisms developing at 37° after a period of apparent quiescence. Later the decrease in bacteria was marked. The Barn milk, after the quiescent period, showed a steady and marked increase in bacteria. On gelatin, so far as the incomplete data show, it appears that much the same conditions obtained with each kind of milk—at first a great increase in numbers of bacteria, followed by a decrease.

The percentage of acid noted in the various samples of milk at the periods of observation are given in Table 9.

TABLE 9.

PERCENTAGE OF ACID OF MILKS HELD AT 0° C.

Date	Age of Milk in Days	Dairy	Barn
8-6 -'08	0	.16	.18
8-12-'08	6	.15	.18
8-21-'08	15	.17	.19
8-26-'08	20	.17	.19
9-11-'08	36	.21	.21
9-11-'08	36	.21	.20
10-20-'08	74	.54	.32
11-20-'08	106	.65	.58
1-13-'09	160	.92	.91
2-25-'09	203	.68	.77

This shows a considerable increase in acidity for each specimen, but no plates with a marked lactic odor or with any considerable number of lactic appearing colonies were encountered. From this it is evident that the lactic acid organisms did not overcome the other organisms and that the acidity to some extent at least was produced by organisms other than lactic.

The distribution of the nitrogen was determined in the specimens brought to the laboratory after 203 days storage with the following results:

TABLE 10.

DISTRIBUTION OF NITROGEN IN THE SPECIMENS HELD AT 0° C.

	Percentage Total Nitrogen	Percentage Insoluble Nitrogen	Percentage Soluble Nitrogen	Percentage of Total Nitrogen Present as Soluble Nitrogen
Barn 0°	.360	.098	.262	72.78
Dairy 0°	.3966	.111	.2856	72.01

The digestion of nitrogenous bodies herein shown can hardly be attributed to the enzymes inherent in the milk itself and must therefore necessarily be attributed to bacterial action. The most peculiar thing about the above results is the marked loss in total nitrogen which can best be explained by assuming a liberation of free nitrogen. This is quite probable because it was observed that on opening the flasks held at this temperature there was a vigorous evolution of gas which was not at all suggestive of ammonia.

Water.—The specimen of water kept at this temperature showed a period of quiescence when first refrigerated similar to that exhibited by the milks, but this persisted for a longer period in the case of the water. The rise was so rapid in the organisms developing on gelatin that no count was obtained for a considerable period because of the low dilutions. The small amount of data at hand evidently show a decrease finally and this decrease is also marked in the organisms developing at 37°.

CONCLUSIONS.

In milks held at −9° C. there was a clumping of the casein and fat, no increase in bacteria developing on agar and gelatin, and an increase in the amount of soluble nitrogen and a decrease in the acidity.

In milks held at 0° C. there was a marked increase in the bacterial content resulting in an increase in acidity, an increase in the percentage of soluble nitrogen so that it eventually amounted to over 70 per cent of the total nitrogen, and a decrease in the total nitrogen content probably due to a liberation of free nitrogen.

This work has especial importance in connection with the handling of one dairy product, viz., cream. The amount of cream received by the city milk dealer does not vary widely from day to day, while the demand for it varies, being influenced by temperature conditions, occurrence of holidays, etc. The storage of cream for considerable periods is therefore practiced. As is shown by the data presented, the temperature of storage, 33 to 40° F., is such as to exclude the growth of the ordinary lactic bacteria, but not the growth of the putrefactive organisms.

The cream may be normal in appearance and taste but it may contain the by-products of the putrefactive bacteria in such amounts as to render it harmful. Poisoning by ice-cream is quite frequent, by milk very rare indeed. The question may be raised: Could not many of the cases of ice-cream poisoning be traced to the use of cream in which, during prolonged storage, toxic products have been formed?

In pasteurized milk the lactic bacteria are usually absent, a free field is left for the putrefactive spore-forming bacteria, whose action on the milk may not be apparent to the consumer, and yet the milk may be distinctly harmful.

In practical effects cold storage is identical with pasteurization since it removes from the sphere of action that class of bacteria that prevent the growth of harmful kinds of organisms, while it allows the harmful bacteria to develop.